

# Chapter 6

## Fluorescence Observables and Enzyme Kinetics in the Investigation of PPI Modulation by Small Molecules: Detection, Mechanistic Insight, and Functional Consequences

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### 6.1 Introduction

Protein homo- or hetero-oligomers are widespread in living systems. In many instances, their functional roles have been established or conjectured [1, 2], and the potential impact of their study on the understanding of apparently unrelated phenomena, such as protein folding and inter-domain interaction, has been underlined [3]. The discovery of small molecules able to modulate protein–protein interactions (PPIs), hence changing the stability of the oligomers, may therefore assume a strong functional significance. However, PPIs often involve extended protein interfaces which have long been perceived as chemically featureless; therefore, designing small molecules with a potential to interfere with high affinity and specificity with a multiprotein complex, and modulate its stability, may represent a challenging chemical problem having functional biological implications [2, 4–7]. Fortunately, the above perception is increasingly refuted [8, 9], and examples of small compounds able to bind to a protein surface with affinities high enough to compete with the binding of the protein to other proteins are rapidly accumulating [2, 4, 6–8, 10 and Chap. 2 in this book]. A qualitative/quantitative characterization of the effects of these compounds on protein–protein complex formation is essential in drug discovery processes targeting PPIs and, as we shall see, has been obtained through several experimental approaches. On the other hand, addressing and characterizing the mechanism of action of such small molecules, including the relevant structural features, often remains a prohibitive task. However, it is this higher level of knowledge that, while answering intrinsically relevant questions related, for example, with the types and number of PPIs involved, may provide valuable hints

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for designing new small molecules characterized by stronger, or more specific, modulating actions [10].

Essentially, any biophysical experimental observable, whether associated with light-matter or electron-matter interactions (absorption, both molecular and plasmonic, emission, scattering, diffraction), with heat exchange, with molecular-size-dependent radial distribution in a rotating centrifuge, or migration in a chromatographic column or under an electric field, can in principle be employed to investigate PPIs, and many of them have actually been so [11–14]. On the other hand, most reported examples of modulation of these interactions by small molecules are based on experimental methods that probe a narrower group of biophysical observables, especially those adaptable for protocols with high-throughput-screening capacities [15]. Designing and setting up an experimental method that is able not only to give a signal when testing an active compound, but also to provide structural and/or functional insight on the effects of its binding on PPIs, that is, on the structure/stability of supramolecular protein assemblies, can be severely challenging. Such a method must fulfill the requirements for detecting and characterizing PPIs, but with a few important additions: It should be sensitive enough to reflect changes in these interactions that (1) may be intrinsically small and (2) are often produced in ligand/protein complexes present at much lower concentrations than the unperturbed protein oligomers. This requirement is particularly severe in the many examples of functional relevance in which transient multimeric protein assemblies are addressed and weak to moderate PPIs that govern their formation are modulated [16]. Finally, and rarely achieved, (3) the experimental approach should also provide mechanistic insight, that is, it should highlight the changes in structure and behavior brought about in the protein assembly by the binding to the small molecule.

In the reported examples of PPI modulation, spectroscopic methods are most often employed for the purpose, being naturally connected with the structure of the molecules probed and the interactions they experience and, at least in many favorable cases, because of their good sensitivity and specificity. Noteworthy exceptions are represented by studies based on size-exclusion chromatography (SEC), sedimentation equilibrium analytical ultracentrifugation, usually in the sedimentation velocity mode of measurement [17], and surface plasmon resonance (SPR). The first two techniques are based on a spatial separation of protein monomers and different multimers and have found some applications in the field of PPI modulation screening (some cases are described in Ref. [18]). However, because of complicating effects (e.g., in SEC, nonspecific binding of differently aggregated proteins to the stationary phase may differentially affect the chemical potentials of monomers and different multimers [19]), and, more generally, because of the competing tendency of the system to locally re-establish the aggregation equilibrium, thus blurring the desired size-based spatial separation, these methods require an optimization of the experimental conditions and a careful calibration and data analysis to yield reliable quantitative data [20, 21]. SPR has become a widespread method to monitor formation/dissociation of protein complexes [22] and, in a few examples, to screen the effects thereon of libraries of

small molecules (see, e.g., Refs. [23] and [24]). In this technique, the rate constants for association and dissociation of an added compound to an immobilized partner are usually evaluated from the time evolution of the observable, and, from their ratios, binding equilibrium constants are estimated [25]. SPR suffers from a few limiting features; for example, irreversibility of ligand/target complex formation and, relevant to our subject, the occurrence of other processes following binding, such as protein–protein complex formation or dissociation, represent undesired events that complicate data fitting. Also, the SPR observable consists in shifts in the resonance wavelength of gold surface plasmons caused by the binding of organic molecules on the metal surface. It is not, therefore, a molecular spectroscopic technique and can hardly provide a molecular-scale insight comparable with that obtainable from spectroscopic tools. Among the latter, methods based on nuclear magnetic resonance (NMR) [16, 26] and on molecular fluorescence (see, e.g., Ref. [27]) are the most powerful and most often employed. Both families of approaches can provide direct insight into the structural details and the dynamics of protein–protein and protein–ligand complexes, though with their specificities, advantages, and limitations. NMR applications are described elsewhere in this volume (Rebecca Del Conte, Daniela Lalli, Paola Turano, NMR as a tool to target PPIs). Here, we have collected and commented on some representative examples of the potential of fluorescence-based methods in the screening and molecular-scale mechanistic investigation of PPI modulation by small molecules (this overview is intended to be by way of example rather than exhaustive).

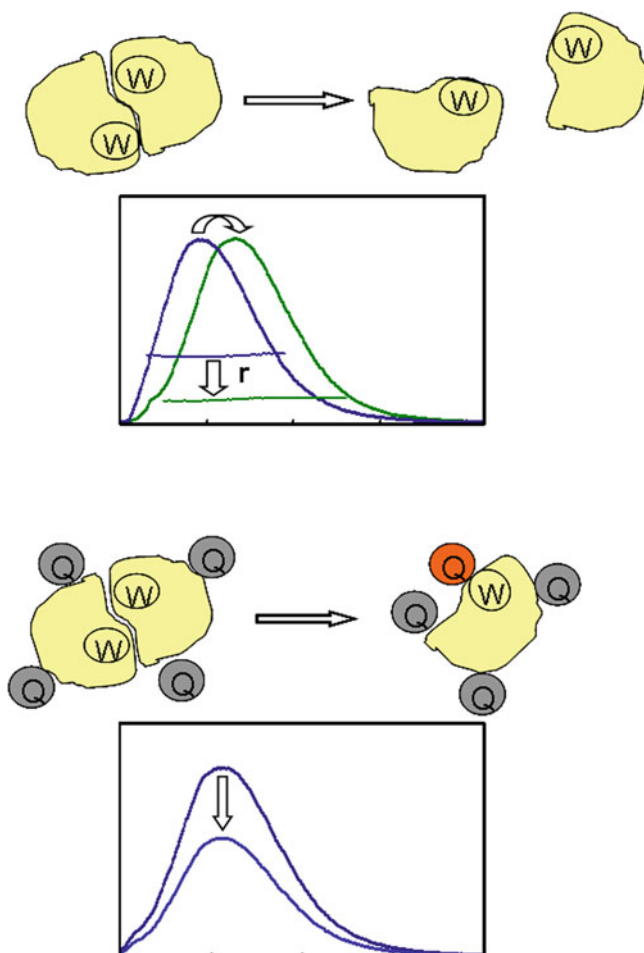
From a quite different point of view, when multimeric enzymes are involved, kinetic analyses can be employed to screen the functional consequences of the modulation of PPIs by small molecules and, more relevant in the perspective of this contribution, to test mechanistic hypotheses. The potential of this experimental opportunity has probably been overlooked. In the final part of this chapter, we will briefly and critically review some relevant examples.

## 6.2 Fluorescence Observables

These experimental approaches take advantage of a variety of observables. Properties such as spectra, intensities (related to quantum yields), time-decays, and anisotropies of intrinsic protein fluorophores, of extrinsic fluorescent tags and, even, of the same small molecules added to modulate PPIs, as well as phenomena such as static and collisional quenching, including electron and excitation-energy transfer, or exciton interaction, whose efficiencies crucially depend on the distance between the partners and their relative orientation [28, 29], may in principle be used to monitor changes in the protein aggregation pattern.

### 6.2.1 Protein Fluorescence

Changes in properties of the intrinsic fluorescence of proteins related with changes in their aggregation state (Fig. 6.1) have been reported, and exploited, in quite a few instances. Pertinent examples are the cases of interferon- $\gamma$  dimer/monomer transition, of calmodulin interaction with a neuronal target protein (see Ref. [29], Chap. 16), of melittin self-association (see Ref. [29], Chap. 17), and of the complexation of a retinal phosphodiesterase subunit with two subunits of heterotrimeric G-protein transducin [30].



**Fig. 6.1** Protein multimer disruption and steady-state intrinsic (tryptophan, W) protein fluorescence. *Top* emission spectral shift associated with a change in W environment; change in anisotropy related with a change in rotational mobility. *Bottom* decrease in emission intensity due to increased accessibility of external quenchers (Q) to Ws

Because of the different electronic distributions of the lowest excited ( $S_1$ ) and ground electronic states, of the possible involvement of  $n\pi^*$  states and of possible free-volume-requiring  $S_1$ -state relaxation processes, emission spectra are often sensitive to the polarity, proticity, and microviscosity of the fluorophore environment. The main intrinsic fluorophore of proteins, tryptophan, exhibits such a sensitivity: Its emission shows a bathochromic shift with increasing polarity of the local environment (upper panel in Fig. 6.1), and a blurring of the vibronic structure when moving from an aprotic to a protic environment, associated with the stabilization of the  ${}^1L_a$  relative to the  ${}^1L_b$  states (see Ref. [29], Chap. 16). Thus, the intrinsic protein emission spectrum can be used to monitor changes in the solvent exposure of the tryptophan residues. In the only example known to us of PPI modulation by small molecules monitored in this way, shifts of the emission maximum of glutamate dehydrogenase enabled a reversible hexamer-to-trimer dissociation of the protein to be observed. The approach, based on dynamic light scattering, aimed at progressively disrupting PPIs using guanidinium hydrochloride at low concentrations. Increases in  $\Delta G$ s were estimated for the process upon binding of norvaline and glutamate to the protein, indicating a ligand-induced stabilization of the hexamer [31], a rare example of positive modulation of PPIs by small molecules.

Measuring changes in intensity of the intrinsic protein fluorescence in complexes relative to the corresponding separated components seems quite a simple way to monitor complex formation or disruption (see, e.g., Ref. [30]). In spite of this, we are aware of only one example of small-molecule-induced changes in protein complexation investigated this way. A combination of SEC and intrinsic protein fluorescence measurement showed that tethered peptides, corresponding to the N- and C-termini of HIV-1 protease, targeted the dimer interface of HIV-1 protease and decreased the fraction of enzyme dimer in solution [32]. Here, the presence of a tryptophan near the monomer/monomer interface was exploited: Addition of the tethered dipeptide inhibitor to the protein caused a marked fluorescence quenching that was not observed with a conventional active-site inhibitor and was presumed to be due to an 'increased solvent exposure' of this tryptophan in the monomers. This statement is probably misleading, as it suggests that exposed tryptophans are more likely quenched than are more buried ones. This is true when accessibility by external quenchers, such as acrylamide or oxygen, is concerned (see Ref. [29], Chap. 16 and the following lines). However, it is well known that the lifetimes and quantum yields of tryptophans in proteins are controlled by a number of quenching processes that involve several different residues, as well as peptide bonds of the backbone [33]. As a result, lifetimes and quantum yields do not correlate with emission maxima, that is, with the solvent exposure of the tryptophans (see Ref. [29], Chap. 16). So, in principle, these observables could be exploited to monitor changes in the aggregation state of the proteins if these result in structural changes that occur in the proximity of tryptophan residues, even buried ones, and that, in turn, affect the efficiency of the quenching processes.

Protein fluorescence quantum yields are reduced in the presence of dissolved quenchers that can access one or more tryptophans. Thus, measurements of protein

emission intensity in the presence of dissolved quenchers can reveal changes in accessibility resulting from formation or disruption of protein complexes (lower panel in Fig. 6.1). For example, increased quenching by KI of the intrinsic fluorescence of *Plasmodium falciparum* triosephosphate isomerase, following mutation of a tyrosine at the subunit interface to glycine, indicated a larger accessibility by the iodide quencher in water of a tryptophan residue near the interface, associated with dimer disruption, as confirmed by gel filtration experiments [34]. In a slightly different approach, the tetramer-to-dimer and dimer-to-monomer dissociation kinetics of three apolipoproteins of the E family were followed by observing the decrease in intensity of the intrinsic protein fluorescence following dilution of the proteins in a solution of acrylamide, a classical tryptophan quencher [35]. In spite of the potential of this fluorescence observable, we are not aware of the use of experiments based on differential accessibility to quenchers to test PPI perturbation by small molecules.

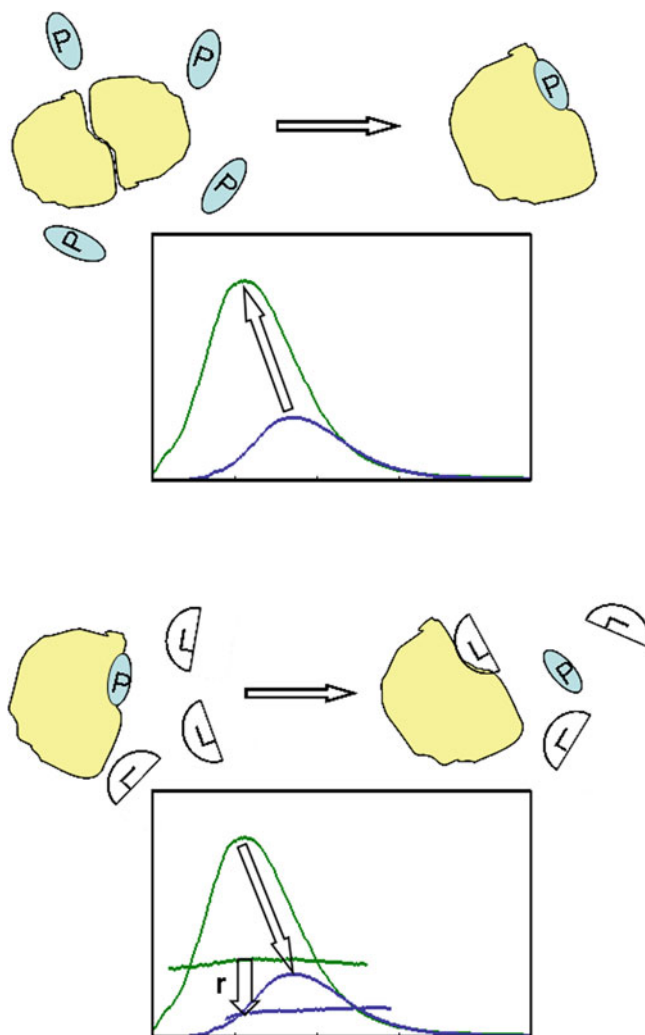
In general, the intrinsic steady-state protein fluorescence properties, most notably anisotropy (upper panel in Fig. 6.1), are little employed to monitor changes in the protein aggregation pattern caused by interaction with small molecules, in spite of the simplicity of these measurements. The small number of examples of this kind might imply that the tryptophan emission properties are rarely significantly affected by changes in the tertiary and quaternary structure of proteins. We are not, however, aware that this has ever been actually observed and explicitly reported.

## 6.2.2 Fluorescence of Probes

When fluorescent labels are employed, the source of information about changes in PPIs, or in protein assemblies, is a change in the probe fluorescence properties. In order to provide information of mechanistic relevance, such changes must be traceable to varied probe environment, accessibility to quenchers, proximity to other fluorophores or rotational mobility.

As an example of a change in a probe environment, a fluorescence assay has been designed to test the binding of a library of tetrapeptides, modeled on the N-terminus of the pro-apoptotic protein Smac, to the surface pocket of the BIR3 binding region of the anti-apoptotic XIAP protein. Here, a solvent-sensitive fluorogenic naphthalene-based dye was attached to a tetrapeptide through a thiol linkage and, upon binding to XIAP, underwent a solvatochromic emission shift and a change in emission intensity (upper panel in Fig. 6.2). These changes, or, more precisely, their reversal (lower panel in Fig. 6.2), were employed to monitor the displacement of the bound tagged peptide by other untagged tetrapeptides, and quantify the corresponding binding equilibrium constants [36].

Anisotropy changes, which reflect changes in rotational mobility of the fluorophore in the free and bound states or, with lower sensitivity, when bound to a protein in different aggregation states, have been employed to characterize small



**Fig. 6.2** *Top* protein multimer disruption and steady-state probe fluorescence: emission spectral shift and intensity change associated with a change in probe environment. *Bottom* reversed spectral changes and decrease in probe emission anisotropy caused by mass-law-governed displacement of the probe by a tested ligand ( $L$ ). Here,  $P$  represents a fluorescent probe or, more often, a tagged peptide with good affinity for the protein–protein binding site

inhibitors of PPIs. An example of the latter kind is provided by self-association of a fluorescein-labeled retinoid-X-receptor to form tetramers that was followed by measuring the fluorescence anisotropy of the probe with increasing protein concentration. In the presence of 9-cis-retinoic acid, the final anisotropy was much lower than in its absence, an indication that formation of the tetramer, characterized by a slower rotational diffusion, was inhibited by this ligand [37]. A similar

fluorescence polarization assay, developed in a high-throughput format, was employed to screen compounds able to perturb the interaction between two peptides, designed from the binding regions of fibronectin and tissue transglutaminase, two proteins whose complex is believed to promote tumor cell adhesion and to be involved in the process of tumor dissemination [38]. One of the two peptides was tagged with a fluorescein molecule; addition of the other peptide resulted in a saturating increase in the anisotropy of the probe, due to complex formation. Small compounds able to inhibit formation of the complex caused a decrease in the observed anisotropy at fixed peptide concentrations.

The most widely employed fluorescence polarization assays are, however, based on competitive displacement of a labeled small molecule, often a peptide, known to bind at a region crucial for PPI (lower panel in Fig. 6.2). Rather than directly monitoring PPI modulation, the assay aims at testing the ability of small molecules to replace the labeled small molecule, which is assumed to mimic the partner protein. For example, some peptides designed from pro-apoptotic Smac were shown to bind to the BIR2 and BIR3 domains of the anti-apoptotic XIAP inhibitor protein [39–41]. This binding was quantitatively characterized by measuring the anisotropy of the emission from a peptide labeled with a carboxyfluorescein, which was progressively displaced from the BIR2 and BIR3 domains of XIAP by the tested unlabeled peptides in dose–response experiments. Similarly, the  $IC_{50}$ s of two small peptide-based inhibitors of the interaction between the von Hippel–Lindau protein (VHL), the substrate recognition subunit of an ubiquitin ligase, and its primary substrate, the hypoxia-inducible factor  $1\alpha$  (HIF- $1\alpha$ ), were determined by measuring the anisotropy of a fluorescein-labeled HIF- $1\alpha$  peptide that binds VHL with a 560 nM affinity [42]. In another example, inhibition constants of several green tea polyphenols versus two Bcl-2 family anti-apoptotic proteins were determined with a competition assay based on dose–dependent displacement of a fluorescein-labeled peptide, reproducing the BH3 domain of the pro-apoptotic counterparts, and measurement of the resulting decrease in emission polarization of the fluorescein probe [43]. Essentially, the same approach has been employed [44, 45], also in a high-throughput version [46], to find inhibitors of the BH3/Bcl-2 interaction, to identify chelerythrine as an inhibitor of the Bcl-XL/BH3 complexation [47], to screen a series of terephthalamides as inhibitors of the Bcl-XL/BAK peptide binding [48], to test molecules, selected using a shape-comparison program, for activity against the ZipA–FtsZ interaction, an anti-bacterial target [49], and to identify a small inhibitor of the interaction between one of the proteins of the 14-3-3 family, implied in physiological and pathophysiological interactions with more than 200 proteins, and the pS259-Raf-1 peptide [50]. The additivity of fluorescence anisotropy [28] was crucial in some of these applications to enable the fractions of labeled peptides, bound and free, and the binding equilibrium constants to be determined. A problem with this kind of assay is that, in order to characterize quantitatively the binding of potent inhibitors, high-affinity labeled peptides, to be replaced by the tested compounds, must sometimes be designed and obtained for the purpose [41]. As a general comment on the widespread methods based on polarization of probe fluorescence, while



these experiments are efficient as bases for medium- to high-throughput screenings (HTS), because they rely on rotational mobility, which is controlled mainly by size and, to a lesser extent, shape, they generally lack structural/mechanistic insight on the protein/ligand binding modes.

Under favorable conditions, H-type exciton coupling between closely associated fluorescent labels may result in a marked emission quenching [51]. This phenomenon was exploited to monitor the dissociation kinetics of a subunit from trimeric tumor necrosis factor  $\alpha$  induced by a small inhibitor of the protein and to deduce conclusions on the mechanism of the process [52]. Dissociation of a subunit caused loss of H-type exciton coupling between fluorescein molecules labeling different subunits and resulted in emission recovery.

A couple of examples of PPI modulation by small molecules are characterized by a hazy description of the molecular bases of the assay employed. In the first one, a change in fluorescence from a probe was only assumed to reflect changes in a protein assembly, but was not interpreted on a molecular level. The fluorescence of dansylated *L. casei* thymidylate synthase (TS) was monitored to investigate structural changes of the dimeric protein upon interaction with a 20-mer peptide designed to reproduce a sequence at the subunit interface [53]. Addition of this peptide, that inhibits TS, was found to result in a decrease in the emission intensity of a dansyl probe specifically bound to a Cys residue which resides at the dimer interface. However, both this quenching and the protein inhibition were attributed to a peptide-induced decrease in spectroscopically and kinetically observable labeled protein in solution due to aggregation/precipitation, rather than to interference of the peptide with protein dimer structure or stability. A similar lack of molecular-scale insight characterizes an affinity-based assay proposed as a screening tool for PPIs [54]. A change in fluorescence intensity of a 'generic probe' upon thermal denaturation of a protein to which it is bound was employed in a high-throughput miniaturized test. An increase in thermal stability was expected and observed as a consequence of the binding of a tested compound to the labeled target protein. This small-molecule binding possibly but not necessarily inhibited binding with other proteins.

A rare example of the use of fluorescence changes to investigate the ability of small molecules to inhibit protein-protein binding is provided by the competition between antimycin A and the pro-apoptotic proteins BAK, BAX, and BIK for binding to the hydrophobic grooves of Bcl-XL [55] or of a recombinant Bcl-2 [56], anti-apoptotic proteins overexpressed in many cancer cells. In these cases, it was an emission enhancement of antimycin A itself, that is, the small inhibitor of protein-protein association, that was employed to demonstrate its binding to the Bcl proteins. The assumption was that the emission quantum yield of this fluorophore is larger in the hydrophobic environment provided by the proteins. Conversely, in competition experiments, a decrease in emission from antimycin A was used to monitor binding of a nonfluorescent methoxy derivative to the same groove. In the second paper, parallel experiments were made with 1-anilino-8-naphthalene sulfonate, a widely employed hydrophobic probe with emission properties that are strongly environment dependent.

Another example of fluorescent small PPI modulator is a fluorene-based compound able to block the interaction between  $\alpha 2\beta 1$  integrin and collagen, an interaction that has been shown to have an important role in thrombus formation and cancer spread [57]. The peculiarity and interest of this example come from the fact that this inhibitor was specifically designed in order not only to bind to the flat collagen-binding domain of the integrin but, also, to be fluorescent. This condition is not, however, sufficient to make a good self-probing small inhibitor. In addition, some property of the fluorescence must change upon binding of the small molecule to the target protein domain. In this case, a strong emission enhancement accompanied the binding, a result not easily predictable. A molecular structure composed of one or more fluorophores connected to a biologically active group through single bonds, which allow for some torsion, is probably a useful structural feature for a fluorophore that is desired to have its emission quantum yield increased in a constrained environment. A similar feature characterizes some well-known fluorescent DNA dyes, for example, Hoechst 33258 [58]. Finally, but importantly, some evidence must be available about the ability of the bound fluorescent molecule to modulate the binding affinity of the protein toward other proteins.

### ***6.2.3 Bimolecular Processes: FRET***

Measurement of the efficiency of fluorescence (or Förster) resonance energy transfer (FRET), a long known powerful method for obtaining information about molecular-scale distances [28], is the most widely employed fluorescence-based method to investigate protein–protein complexation equilibria. Typically, selective excitation of an excitation-energy donor results in emission from an acceptor with an efficiency that depends on distance and relative orientation. Measurement of this efficiency for a well-characterized donor–acceptor pair enables distance between the partners to be estimated under reasonable assumptions on their relative orientation. FRET between fluorescent partners, including proteins, can be quantitatively assessed both in steady-state and in time-resolved (TR) experiments [28, 59]. In the former, the donor and acceptor emissions are measured under continuous excitation, either as full spectra or, in higher-throughput screenings, as intensities at selected excitation and emission wavelengths. Steady-state FRET experiments may be employed to investigate the association/dissociation kinetics of multimeric proteins when these kinetics are slow relative to mixing/dilution times [35]. TR experiments consist in acquiring the fluorescence time profiles following pulsed excitation and analyzing them to derive FRET efficiencies from changes in fluorescence intensity decays (donors) or rises (acceptors). FRET experiments can also be performed on living cells by combining steady-state or TR fluorescence measurements with the spatial resolution of a conventional optical or a confocal microscope [28, 60]. As one of the many examples of experiments designed to monitor PPI in cells, steady-state FRET between two different mutants

of GFP fused to two human Four-and-a-half LIM-only proteins, FHL2 and FHL3, was employed to determine their interaction and to locate the site of this interaction in a single intact mammalian cell [61].

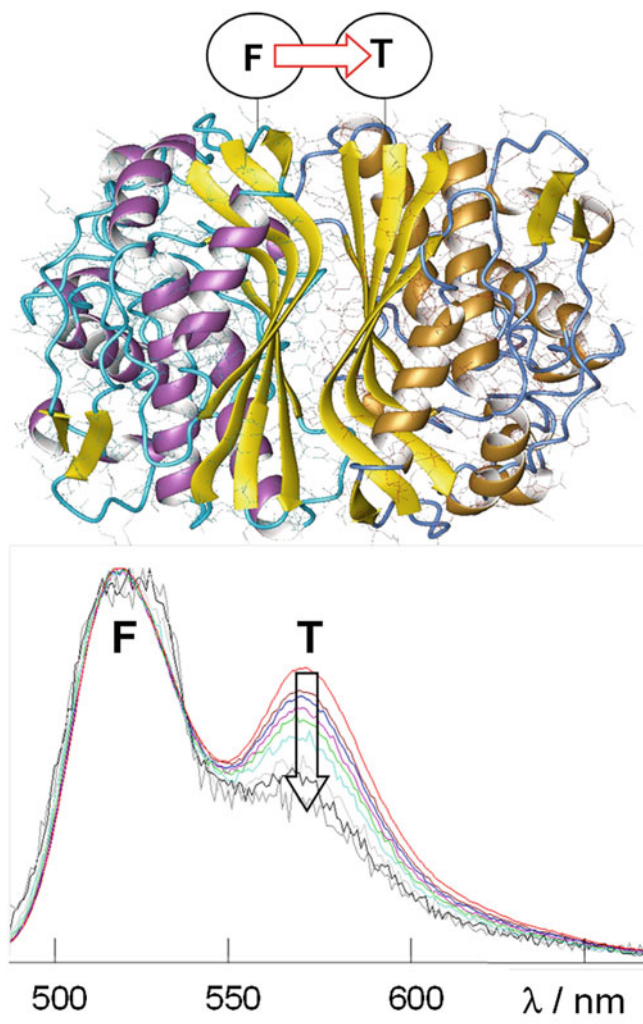
There follows a selection of examples of FRET-based experiments, designed to monitor modulation of PPIs by small molecules.

FRET from fluorescein to tetramethylrhodamine, selectively bound to two cysteine residues, each on a different monomer of dimeric human TS (Fig. 6.3), was exploited in concentration-dependent steady-state fluorescence measurements to determine the fraction of dimeric protein at each total protein concentration and, as a result, the monomer/dimer equilibrium constant [62]. The assay was then used to test whether some octapeptides, found to inhibit hTS through an unconventional mechanism, were able to disrupt the protein dimer [63]. The experimental results showed only a minor perturbation of FRET, consistent with the crystallographic evidence of a binding of the peptides at the subunit interface without causing significant destabilization of the protein dimer.

A small library obtained by computational interrogation of the binding pocket of protein S100A10 was screened to identify compounds able to destabilize the complex between S100A10 and the phospholipid-binding protein Annexin A2 [64]. Steady-state FRET from Cy3-labeled Annexin A2 and Cy5-labeled S100A10 was employed in the screening. In this case, S100A10 protein labeling was not site directed, and its stoichiometry was only roughly defined. So, while fairly easily achieved, such an approach can only provide semiquantitative information on the efficacy of the tested small compounds in destabilizing the protein association.

FRET between two fluorescent probes, bound to the antibodies for two different epitope tags linked to the two monomers of HIV-1 integrase (IN), earlier used to characterize the monomer/dimer equilibrium of this protein [65], has later been combined with an equilibrium analysis of a binding model for IN-IN interaction, including the monomeric and several oligomeric species in the presence of an IN-dimer ligand. Dithiothreitol and  $\beta$ -mercaptoethanol weakened the IN monomer-monomer interaction. On the other hand, two peptides derived from LEDGF, a cellular cofactor that interacts with the IN-dimer interface, and a small molecule, all of which compete with LEDGF for binding to IN, were found to increase the stability of the IN dimers [66].

A nice example of the use of FRET, in combination with fluorescence microscopy, to monitor the effects of small compounds on PPIs in living cells is provided by the investigation of the effects of compounds, previously found able to disrupt BH3 interactions *in vitro*, on the heterodimerization of Bcl-XL with the pro-apoptotic proteins, BAX and BAD [46]. Intact cells were co-transfected with the expression vectors BAX fused to yellow fluorescent protein (YFP) and Bcl-XL fused to cyan fluorescent protein (CFP). Co-transfection resulted in an increase in the YFP-to-CFP emission ratio, relative to separately transfected cells, due to CFP-to-YFP energy transfer. The addition of the above compounds caused decreases in this 'FRET ratio' consistent with the activities of the compounds *in vitro*. The same approach, only involving different FRET donor and acceptor,



**Fig. 6.3** FRET from fluorescein (*F*) to tetramethylrhodamine (*T*) bound to Cys 43 and 43' of the human thymidylate synthase dimer. FRET efficiency is correlated with the relative T/*F* emission intensity and decreases with decreasing total protein concentration, from ca 300 to ca 5 nM [ 62]

was employed to test the effects of the same compounds on heterodimerization of BAD with either Bcl-2 or Bcl-XL in intact cells.

Changes in the efficiency of homo-FRET, that is, excitation-energy transfer between a donor and an acceptor of the same chemical nature, such as two tryptophan residues or two extrinsic, identical probes in a protein oligomer, including homo-oligomers, may reveal changes in the protein oligomerization state [67]. Here, it is the depolarization associated with excitation-energy transfer between like fluorophores that is usually measured [28]. As a clever example of the use of

homo-FRET to investigate the effect of small molecules on protein oligomerization, we mention the case of the serotonin<sub>1A</sub> receptor whose oligomers are potentially implicated in the functional roles of the protein. Homo-FRET and fluorescence lifetime measurements have been used to monitor such an oligomerization in cells expressing the serotonin<sub>1A</sub> receptor tagged to enhanced yellow fluorescent protein [68]. The emission anisotropies were found to be lower than the value predicted for the monomeric protein, and the depolarization was attributed to homo-FRET within protein oligomers. To support this assignment, the fluorophores were progressively photobleached. Because the efficiency of homo-FRET correlates with the spectral absorption/emission overlap, the bleaching led to recovery of anisotropy. This was analyzed versus predicted recoveries for collections of dimers, trimers, and higher aggregates. This analysis, combined with the extrapolated anisotropies at full fluorophore photobleaching, enabled the authors to discriminate between oligomers of different sizes. In particular, they tested the effects of some known agonist and antagonists of the serotonin receptor and found that while treatment with an antagonist (p-MPPI) lowered the fraction of higher-order oligomers, the agonist (serotonin itself) induced the formation of higher-order oligomers. Homo-FRET combined with microscopy, and, in some instances, with the increased selectivity afforded by two-photon excitation, has recently provided subcellular resolution imaging of protein oligomers [69, 70].

Conventional, steady-state FRET measurements may suffer from limited accuracy because of interfering emissions, particularly from complex biological samples. An impressive increase in sensitivity has been obtained by employing long-lived fluorophores, mostly lanthanide ions, to enable time-gated measurements of the donor/acceptor signals delayed, with respect to excitation, from several microseconds to a few milliseconds, that is, a time when all background, usually nanosecond, emissions have decayed [71, 72]. Biosensors based on nanocrystals doped with lanthanides have been proposed for this application [72]. A similar increase in analytical robustness is obtained using a bioluminescent excitation-energy donor, typically, a luciferase (BRET, [13]). Here, intensities are extremely low, but interferences are essentially absent, as no excitation light is required. The methods are well suited for medium-to-high throughput screenings. An example of the first approach, often, and rather confusingly, called time-resolved FRET (TR-FRET), consists in the screening of 1,280 compounds to identify inhibitors of the dimerization of a 106-residue domain of the capsid protein of hepatitis C virus [73]. The Core-106 fragments were tagged with an N-terminal glutathione-S-transferase (GST) or Flag peptide. Europium cryptate, a long-lived donor, and allophycocyanin were used to label anti-GST and anti-Flag antibodies. Association between GST-core106 and Flag-core106 was assessed by measuring FRET between the two fluorophores following antigen/antibody recognition. Another example is provided by the discovery of potent, nonpeptide inhibitors of the interaction between leukocyte function-associated antigen-1 (LFA-1), a member of the  $\beta_2$ -integrin family of adhesion molecules, and intracellular adhesion molecule ICAM-1 [74, 75]. In this case the strategy consisted in immobilizing one of the two partners, tagged with a fluorescent probe, and adding the other partner, tagged with a long-lived europium

luminophore using the biotin/streptavidin recognition. The decrease in FRET observed in the presence of screened compounds was a measure of the ability of the latter to disrupt the LFA-1/ICAM-1 interaction. Several more HTS TR-FRET assays differing only in some details, say, the nature of the donating lanthanide and the accepting fluorophores or the strategy to tag the interacting proteins with the two fluorophores, have been applied to search for small PPI inhibitors. An  $\text{Eu}^{3+}$  cryptate-conjugated anti-FLAG antibody and an anti-6His antibody conjugated to a fluorescent excitation-energy acceptor have been employed in an assay designed to screen approximately 15,000 compounds to find inhibitors of the complexation of FLAG-fused  $\text{IKK}\beta$  with NEMO-6His, a process involved in inflammatory and autoimmune disorders [76]. A  $\text{Tb}^{3+}$  chelate and Alexa Fluor 488 have been chemically conjugated, respectively, to the G protein,  $\text{G}\alpha_o$ , and its regulator protein, RGS4, and used in a TR-FRET screening of approximately 40,000 compounds to find two inhibitors of this PPI [77]. An europium-labeled anti-His antibody and a streptavidin-conjugated APC fluorophores were employed to label the 6His-apoRBP4 and the biotinylated human TTR proteins to test small compounds that were found to either increase or decrease the affinity of the retinol-binding protein, RBP4, for transthyretin, TTR [78].

### ***6.2.4 Multiple and Other Fluorescence Observables***

The above-mentioned fluorescence observables may be usefully combined within the same investigation. An example is provided by the screening of 60 compounds that had been previously selected by computational methods as possible inhibitors of the down-regulation of the p53 tumor suppressor protein caused by interaction with the calcium-binding protein, S100B [79]. Complexation of the latter with the small compounds, leading to p53 activity increase, was monitored by four different titration experiments: direct measurement of emission changes from the fluorescent compounds due to subsequent additions of the S100B protein; quenching of tyrosine emission from the protein upon titration with the compounds; measurement of tryptophan emission restoration in competition titrations of wild-type S100B into solutions of the complexes of an S100B tryptophan mutant with the small compounds; and measurement of fluorescence from a peptide derived from p53 (F385W) that binds holo-S100B in competition titrations of the small molecules to the S100B-p53 complex.

Another example of combination of fluorescence observables is provided by the screening of small-molecule inhibitors that interfere with the cytohesin-catalyzed GDP/GTP exchange on a truncated version of ARF1, an adenosine diphosphate ribosylation factor ( $\text{N}\Delta 17\text{ARF1}$ ) and/or with the interaction between  $\text{N}\Delta 17\text{ARF1}$ -GTP and its effector protein GGA3 [80]. The two proteins were fused to the fluorescent proteins CyPet and YPet, respectively. To identify the two kinds of inhibitors, the nucleotide exchange on  $\text{N}\Delta 17\text{ARF1}$  was monitored in real time by measuring the associated enhancement of its intrinsic tryptophan fluorescence,

while association of the two proteins was simultaneously monitored by measuring the CyPet-to-YPet FRET: the two phenomena increased with similar rates, suggesting GDP/GTP exchange to be rate limiting. As often found, applications of the first assay were limited to the tested compounds that did not act as inner filters for tryptophan excitation, that is, that absorbed negligibly at 280 nm.

Among the fluorescence observables, some have not been employed to investigate the action of small molecules able to interfere with PPIs. A remarkable example is provided by fluorescence cross-correlation spectroscopy (FCCS, [81]). FCCS is a powerful tool to monitor protein–protein and protein–DNA interactions, both in solution and in cells. Measurement of cross correlation between the time-fluctuations of the emissions from two different, independently excited probes, each attached to a partner of the interacting pair (or larger assembly), provides information on the complex dynamics and thermodynamics. The FCCS approach suffers from difficulties related with probe binding to the interacting partners, especially for monitoring in cells. The problem, however, has now been solved in many cases by employing different strategies, including autofluorescent labeling, that is, expression of the protein of interest fused with different fluorescent proteins, specific chemical labeling, use of fluorescent antibodies [81], or by employing two-photon excited intrinsic protein fluorescence [82]. The method, now implemented on commercial fluorescence microscopes, is therefore recommended for monitoring perturbation of PPIs by small molecules.

While TR emission from probes has found applications (some are quoted in the previous paragraphs), measurement of the time-course of intrinsic protein emission to monitor changes in the aggregation state is apparently an unexplored opportunity. There are a number of practical reasons that make this kind of experiments poorly apt for medium/HTS: Instrumentation is often expensive, measurements are usually time-consuming, and analysis of the results may be rather complex [83]. While these observations are intimately related with the interactions experienced by tyrosine and tryptophan residues in the protein, and probably reflect even subtle structural changes with an unprecedented sensitivity, a structural/dynamic interpretation of the changes observed in the time-course of a protein emission remains a difficult task (Ref. [29], Chap. 17). However, because of the wealth of information buried within, efforts have been made, and are currently underway, to set up tools and knowledge able to extract this information [33, 83, 84]. Therefore, it is easy to predict TR intrinsic protein fluorescence to become a major source of structural/mechanistic information on PPIs and their modulation.

### 6.3 Dissociative Inhibition Kinetics

While not directly monitoring PPI perturbations by small molecules, whenever a catalytic efficiency depends on some protein multimeric assembly, kinetic analysis can provide direct evidence of the mechanistic consequences of such perturbations. The key observation in these studies is a modulation by small ligands of the

dependence of specific enzyme activity on protein concentration, that is associated with a mass-balance-law-governed distribution of the protein monomers and various multimers. This effect of added small molecules results from their interfering with the interactions between enzyme subunits usually leading to destabilization of the multimeric assembly.

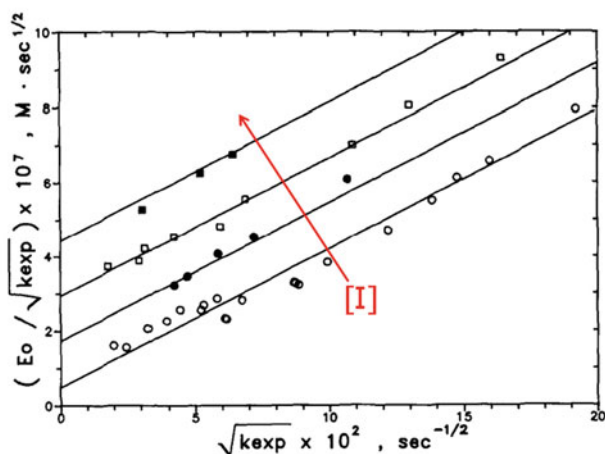
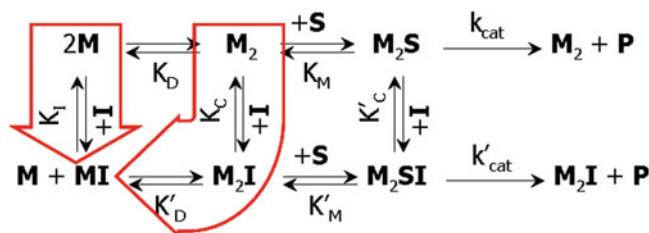
A few different inhibition models of multimeric enzymes have been proposed that involve destabilization of protein–protein attractive interactions. The inhibitor may bind to some protein sequence that is only exposed during the folding process and thus prevents protein association during folding. In an example of this kind, peptides mimicking one or two  $\beta$ -strands from the human immunodeficiency virus 1 (HIV1) interface were shown to inhibit the dimeric enzymes, HIV1 and HIV2 proteases [85]. A standard kinetic analysis indicated a noncompetitive inhibition mechanism, with, however, no hint at the dimeric nature of the enzyme, or at the possibly dissociative character of the inhibition.

For dimeric enzymes, the analysis of the so-called dissociative inhibition model has been provided in Ref. [86]. An inhibitor of a functionally obligate dimeric enzyme was assumed to bind the dimer (competitive inhibition), the monomer (dissociative inhibition), the dimer-substrate complex (uncompetitive inhibition), or both the dimer and the dimer-substrate complex (noncompetitive inhibition, Fig. 6.4). Resolution of the kinetic scheme in the rapid equilibrium regime led to the expectation that  $E_0/\sqrt{k_{\text{exp}}}$  versus  $\sqrt{k_{\text{exp}}}$  plots— $E_0$  being the total enzyme concentration that was varied in the experiments and  $k_{\text{exp}}$  the ratio of the initial reaction rate and the total substrate concentration, which was kept constant—were linear with constant slopes and increasing intercepts at increasing inhibitor concentrations ('Zhang–Poorman plots'). From the best-fit slopes and intercepts, the relevant parameters of the kinetic model were obtained, including the affinities of the inhibitor for the monomeric and the dimeric enzyme,  $K_I$  and  $K_C$ , respectively, and the monomer/dimer dissociation constant of the enzyme,  $K_D$ . The authors applied their analysis to demonstrate that a tetrapeptide corresponding to the COOH terminal segment of HIV-1 protease was, indeed, a dissociative inhibitor, that is, it bound to the inactive monomers (M) and prevented their association into the active dimer ( $M_2$ ).

In the original model, the inhibition was studied under first-order conditions, that is, the total substrate concentration, [S], was assumed much smaller than  $K_M$ . An alternative solution of the kinetic scheme has been recently obtained without making this assumption, in order to extend the analysis to cases in which fulfillment of this condition would require very small [S] values and the need to measure prohibitively slow reaction kinetics [18].

In the recent literature, conformation of kinetic data to this model, as judged from the linearity of the Zhang–Poorman plots and a significant dependence of the intercepts on inhibitor concentrations, has been shown in a number of examples, many of which concern HIV1 protease dissociative inhibitors. To mention a few, dissociative inhibition was found with some nine-residue peptides obtained through an impressive genetic-selection approach [87], with a 27-residue peptide designed from domains at the N- and C-termini of the same enzyme [88], with





**Fig. 6.4** Top kinetic scheme for inhibition of an obligatory dimeric enzyme (adapted from Ref. [86]). Dissociative inhibition is represented by the  $M \rightarrow MI$  and/or the  $M_2 \rightarrow M_2I \rightarrow MI$  paths (red large arrows). Bottom the dissociative inhibition fingerprint:  $E_0 / \sqrt{k_{exp}}$  versus  $\sqrt{k_{exp}}$  plots are parallel lines with intercepts that increase with increasing inhibitor concentration, and the  $[I] = 0$  line has a non-null intercept (taken from Ref. [86]). The meanings of the symbols are given in the text

some interface peptides cross-linked at their amino termini [89, 90] and at side chains [91], with tetracyclic triterpene schisanlactone, a natural product isolated from a fungus [92], and with some naphthalene- and quinoline-based nonpeptidic ‘molecular tongs’ [93–95]. The above kinetic analysis was corroborated by analytical ultracentrifugation results to characterize some alkyl tripeptides as dissociative inhibitors of the same enzyme, both wild type and mutated [96]. Dissociative inhibition seems to be a useful strategy also versus two other HIV-1 enzymes, reverse transcriptase and IN [97].

Among the fewer examples concerning other dimeric enzymes, we mention the inhibition of dimeric aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) by a small compound, Cappsin 1 [98], and that of 3C-like proteinase of severe acute respiratory syndrome coronavirus by some octapeptides derived from the protein N-terminal [99]. Apparently, no available examples involve more-than-dimeric enzymes, and the dissociative inhibition model has not been extended to higher oligomers than dimers.

Of some interest is the mechanistic issue concerning whether a supposed inhibitor of a dimeric enzyme preferentially binds the enzyme monomer, thus preventing its association with another monomer to obtain the active dimer, or binds the already formed dimer causing its disruption. The two paths may require quite different molecular properties for an efficient inhibitor, and, as a consequence, different molecular design strategies. The kinetic model in Fig. 6.4 encompasses both mechanistic routes, that we may simplify as, respectively,  $M \rightarrow MI$  and  $M_2 \rightarrow M_2I \rightarrow MI$ , and characterizes them through the corresponding equilibrium constants,  $K_I$  and  $K_C K_D'$ . Should one path be much slower than the other and the corresponding equilibration not attained in the experimental runs, a more sophisticated kinetic analysis involving all the relevant rate constants and more detailed TR experimental information would likely be necessary to conclude which of the two kinetic paths proposed remains the only functionally relevant one.

Lastly, we remark that dissociative inhibition is not the only mechanism by which a small molecule can inhibit a multimeric obligate enzyme. Kinetic analysis, in combination with crystallographic and calorimetric evidence, showed that inhibition of *Trypanosoma cruzi* triosephosphate isomerase was caused by a small molecule whose binding triggered evolution of the dimeric protein toward an inactive conformation, rather than to dimer disruption. The nonlinear dependence of pseudo-first-order constants of inactivation on inhibitor concentration provided information on the complex inhibition mechanism [100]. As briefly reported in paragraph 2.3, use of FRET between probes enabled the authors to rule out a dissociative mechanism to interpret inhibition of dimeric human TS by some octapeptides designed from a sequence in the inter-monomer surface [63]. Experimental evidence, kinetic, crystallographic, and calorimetric, led to the conclusion that the peptides selectively bound to a dimeric, inactive conformation of the protein, thus stabilizing it. A specific kinetic scheme was solved under the usual fast equilibrium assumption and was shown to be consistent with the observed noncompetitive kinetic behavior with, however, modified interpretations of the plot slopes, intercepts, and crossing point.

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